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Rapid quantification of microRNAs in plasma using a fast real time PCR system

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Abstract

The ability to rapidly detect circulating small RNAs, in particular microRNAs (miRNAs), would further increase their already established potential as biomarkers in a range of conditions. One rate-limiting factor is the time taken to perform quantitative real time PCR amplification. We therefore evaluated the ability of a novel thermal cycler to perform this step in less than 10 minutes. Quantitative PCR was performed on an xxpress® thermal cycler (BJS Biotechnologies, Perivale, UK), which employs a resistive heating system and forced air cooling to achieve thermal ramp rates of 10 °C/s, and a conventional peltier-controlled LightCycler 480 system (Roche, Basel, Switzerland) ramping at 4.8 °C/s. The threshold cycle (Ct) for detection of 18S rDNA from a standard genomic DNA sample was significantly more variable across the block (F-test, $p=2.4x10^{-25}$) for the xxpress (20.01±0.47SD) than the LightCycler (19.87±0.04SD). RNA was extracted from human plasma, reverse transcribed and a panel of miRNAs amplified and detected using SYBR green (Kapa Biosystems, Wilmington, Ma, USA). The sensitivity of both systems was broadly comparable and both detected a panel of miRNAs reliably and indicated similar relative abundances. The xxpress thermal cycler facilitates rapid qPCR detection of small RNAs and brings point-of care diagnostics based upon circulating miRNAs a step closer to reality.

Method summary

We describe a quantitative PCR platform which enables faster ramping between temperatures than conventional peltier-based systems, thereby reducing the time required to complete a PCR reaction. This is particularly important for the development of clinical biomarkers for acute conditions and the feasibility of detecting miRNAs in plasma is demonstrated.

Introduction

PCR is ubiquitous throughout the life and medical sciences and a reduction in the time required to complete a PCR reaction would therefore be of immense benefit. Whilst the choice of fast enzyme is important for the optimization of fast PCR-based systems(1), (2), the speed at which the temperature of the sample can be altered during thermal cycling is the primary rate-limiting factor (3). Using prototype systems many investigators have demonstrated that rapid thermal cycling is possible (4), (5), and under extreme conditions can be completed in less than one minute (6)! The predominant format of existing thermal cyclers comprises a 96 or 384 well block, the temperature of which is controlled by a peltier-based system limited to ramp rates of approximately 4 °C/s. Several quantitative PCR systems which employ rapid thermal cycling are available commercially, but these are based on glass capillaries (LightCycler, Roche) (7) or plastic tubes placed in a centrifuge (Rotor-gene, Qiagen, Crawley, UK). An alternative rapid plate-based approach, which can be more easily integrated into existing workflows, has now been developed. The xxpress® thermal cycler (BJS Biotechnologies) employs resistive heating and forced air cooling to enable ramp rates of up to 10 °C/s.

One of the main applications of PCR is for the quantitation of RNA targets. Following reverse transcription into cDNA, amplification of targets is detected by incorporation of a double-stranded DNA binding fluorescent dye (principally SYBR green) or use of a sequence-specific probe-based system e.g. Taqman. This quantitative reverse transcription PCR (RT-qPCR) approach can be modified to measure small RNAs, specifically miRNAs. Following the discovery that miRNAs exist in a stable form within blood (8)(9)), their potential as biomarkers was soon realised. MiRNA expression patterns characteristic of cancer, cardiovascular disease, diabetes, Alzheimer's and many other conditions have now been reported (10)(11), (12, 12). Typically global miRNA profiles are initially assessed in a discovery cohort using microarrays or deep sequencing and selected informative miRNAs are subsequently measured in a larger population. The method of choice for measuring a defined, diagnostic panel of miRNAs is RT-qPCR.

To perform RT-qPCR, RNA must be extracted from plasma or serum, reverse transcribed and target miRNAs amplified in individual PCR reactions. This process takes several hours and is sufficient for the current applications of circulating miRNA biomarkers for which results may be reported within days or weeks. However, a more rapid assay would facilitate point of care diagnostics and expand potential uses. For example, changes in miRNAs have been associated with cardiac disease (10)), and the ability to measure them in plasma quickly might provide an earlier biomarker for diagnosis of myocardial infarction and ensure more timely therapeutic intervention (13). We therefore assessed the ability of the xxpress system to quantify miRNAs in plasma using high speed cycling in comparison with an existing qPCR system (LightCycler480, Roche).

It was possible to amplify RNAs from plasma cDNA in less than 10 minutes using the xxpress system, compared with ~40 minutes with the LightCycler. However, although the performance of both systems was broadly comparable, variability across the plate and between replicate samples was greater in the xxpress.

Materials and Methods

Oligonucleotide synthesis

All oligonucleotides (Integrated DNA Technologies, Leuven, Belgium) were reconstituted as 100 µM stocks; DNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), RNA in nuclease free water, and stored at -80 °C. Sequences of the RNA miRNA mimic, Reverse transcription oligo, 18S and miRNA primers are shown in Table 1.

qPCR instruments

The xxpress system (Figure 1) employs resistive heating and forced air cooling to facilitate rapid changes in temperature. PCR reactions are performed within proprietary ‘xxplates’ which comprise a

metal base fused with plastic wells, meaning that the sample is only 10 µm from the heat source (Figure 1A). To enable thermal uniformity across all the samples to within ±0.3 °C during holds at a static temperature or within ±0.8 °C during fast ramping (10 °C/s), the xxpress uses an array of infrared sensors to determine the temperature of the test samples and a control algorithm adjusts the heating patterns at a rate of 100 times per second. The xxplates to be analysed using the xxpress system were prepared as follows. Plates were sealed using xxpress compatible polarised sealing strips heated at 170 ^{o}C for 1.5 s using the heat sealer provided (Figure 1B). They were then centrifuged at 1000 rpm for 1 minute in dedicated holders in the centrifuge provided before loading into the xxpress cycling unit (Figure 1B). The LightCycler 480 system with a 384 well block installed has a thermal uniformity of ±0.4 °C within 60 s of target attainment (72 °C). PCR plates (Roche) to be analysed were prepared following the manufacturer's instructions (LightCycler 480 user manual). In brief, plates were sealed using the manufacturer's sealing strips, ensuring all wells were securely covered, followed by centrifugation at 1200 rpm for 2 minutes. Plates were then loaded into the LightCycler.

PCR conditions and data analysis

All PCR reactions were performed for 40 cycles with SYBR Fast qPCR Mastermix (final concentration 1X, Kapa Biosystems, 1 µl DNA or cDNA template, 5 µM forward and reverse primers) in a final volume of 5 µl. The cycling conditions used for amplification of 18S rDNA are shown in Table 2 and employed the same ramp rate on both machines (4.8 °C/s). 18S rDNA amplification used 1 µl of genomic DNA as template (2.5 ng/µl) either neat, or serially diluted in PCR grade water. The same conditions were used for detection of miRNAs, but with a reduced annealing/extension temperature of 55 °C and with ramp rates increased to 10 °C/s on the xxpress. Raw fluorescence data were analysed by baseline normalisation and Ct values were called in accordance with the data analysis software provided on each instrument (threshold set using the second derivative maximum algorithm on the LightCycler and manually on the xxpress).

RNA templates

An RNA oligonucleotide with the sequence of miR-21 and a polyA tail (miRNA mimic) (Table 1) was reverse transcribed using 5 pmol of oligodT-RACE primer; the template was denatured at 50 °C for 5 minutes, then cooled to 4 °C for addition of SuperScript III (Life Technologies, Paisley, UK) followed by incubation at 40 °C for 60 minutes. Total RNA including small RNAs was extracted from 200 µl of human plasma using a miRNeasy Serum/plasma kit according to the manufacturer's protocol (Qiagen, Crawley, UK) and polyadenylated for 1 hour at 37 °C in a 25 µl reaction containing 2 U poly(A) polymerase, 2.5mM $MgCl_2$ and 1mM ATP (Ambion, Life Technologies). A 5 µl volume of polyadenylated RNA was then incubated at 65 °C for 5 minutes with 0.5 µg oligo(dT)-RACE primer and 1µl 10 mM dNTPs in a reaction volume of 8 µl. Following addition of 200 U SuperScript III, 1 U RNaseOut, 1 µl 0.1 M DTT and 4 µl 5X RT buffer, the reaction was incubated at 50 °C for 1 hour followed by 70 °C for 15 minutes.

Ethics

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants/patients were approved by the Research Ethics Committee of the School of Medicine and Dentistry, Queen's University Belfast (Ref:11/05v3). Written informed consent was obtained from all participants

Results and Discussion

To assess consistency across the plate, a single mastermix for amplification of a 155 bp amplicon from 18S rDNA was aliquoted into 48 wells distributed evenly across each type of plate (Suppl. Figure. 1). Amplification was performed using the same cycling conditions on both systems (Table 2). The mean Ct values were very similar from the xxpress (20.01±0.47SD) and the LightCycler (19.87±0.04SD), although the LightCycler was significantly lower (t-test, p = 0.035). However the

xxpress exhibited significantly greater variability than the LightCycler, which generated extremely consistent Ct values (F-test, $p=2.4x10^{-25}$) (Figure 2). Although the performance of the xxpress was less robust, it could still be used in situations requiring rapid detection of large variations in expression between samples (with the inclusion of appropriate replicates). The spread in Ct values on the xxpress was in part linked to the position of the sample on the xxplate, with adjacent wells tending to vary from the mean Ct value in the same direction. The greater variation observed with the xxpress may not be due to poorer temperature control across the plate, but could be related to the optical system, which is amenable to improvement in subsequent versions. To compare the sensitivity of the two platforms, highly abundant 18S rDNA was amplified from a dilution series of genomic DNA. The mean Ct values at each template concentration were not significantly different between platforms (t-test, $p>0.05$). In both cases the Ct values were highly correlated with template concentration (LightCycler, $R^2 = 0.9547$; xxpress, $R^2 = 0.9878$), although again there was a significantly greater variation in Ct values for replicates in the xxpress at several concentrations (Figure 3A).

The method described by Shi and Chiang (14)), was then used to detect miRNAs. Total RNA, including miRNAs, is polyadenylated and then reverse-transcribed with a poly(T) adapter into cDNAs. These are subsequently amplified using a miRNA-specific forward primer and a sequence complementary to the poly(T) adapter as the reverse primer. Initially we tested the ability of each platform to detect a miRNA mimic synthesised with a polyA tail already incorporated (thereby removing any variability associated with the polyadenylation reaction). The limit of detection, as estimated from the linear portion of the standard curve, was approximately $1x10^6$ RNA template molecules for both systems (Figure 3B). Given the high concentrations of specific template miRNA used in this assay, a better signal may be obtained using cell-derived RNA as template (15)).

Our ultimate aim is the rapid detection of miRNAs from blood as biomarkers of cancer and other diseases. As a step towards this we measured the expression of several miRNAs in RNA extracted from three human plasma samples; miR-21, an ‘oncomir’ which plays a role in many types of cancer (16), (17),(18)and heart disease (19)) and has been reported to be elevated in blood from cancer patients (20), (21), miR-22, which has is also differentially expressed in various types of cancer (22)),

175 miR-126, which is involved in regulation of angiogenesis (23)) and miR-486, which is down-
176 regulated in lung cancer and proposed as a biomarker for detection of lung cancer in plasma samples
177 ((24), (25)). The results of quantification of these four miRNAs following forty cycles of PCR,
178 completed in less than 10 minutes with the xxpress compared to 40 minutes using the LightCycler, are
179 presented in Figure 4. The relative abundances of the miRNAs detected by both systems were similar,
180 albeit with greater variation between technical replicates on the xxpress. MiR-21 was the most
181 abundant (lowest Ct) and miR-22 the least abundant (highest Ct) miRNA for all samples on both
182 platforms. Significant differences between platforms in Ct values were observed for many miRNAs
183 amplified from the same sample (e.g. miR-126 in plasma sample 2, $p < 0.001$). This is likely due to the
184 combined effects of differences in the sample temperatures achieved during cycling, the detection
185 systems and performance of the individual assays under different conditions. Another class of small
186 RNA, a Y RNA fragment (hY4-3p) recently shown to be present in plasma (26), (27, 28) was detected
187 consistently by both platforms (ΔCt between the two platforms across three plasma samples was 2.3
188 ±0.3SD) and could potentially provide a stable reference gene.

189 Faster PCR can be achieved by reducing hold times and although this can reduce sensitivity and
190 increase variability (29)) , enzymes are now available from a range of manufacturers which perform
191 well in fast PCR. All data reported were generated with SYBR fast qPCR Mastermix (Kapa
192 Biosystems) but 18S rDNA failed to amplify using Quantitect (Qiagen) on the xxpress, so users
193 should be aware that rigorous optimisation of different mastermixes is required for fast PCR. To
194 facilitate development of rapid assays incorporating fast PCR pre-processing steps must be minimised
195 and there is therefore a growing need to develop enzymes resistant to contaminants and inhibitors
196 present in a range of biological sample materials, such as soil, water and blood (30)).

197 Although many prototype rapid PCR systems have been reported with extremely fast ramp rates and
198 miniaturisation (5), (6), (31)) very few proceed to commercial release. Therefore the xxpress rapid
199 thermal cycler, particularly given its standard block-based design, offers a unique opportunity for the
200 wider molecular research community to adopt fast PCR. The potential applications of fast PCR in
201 situations which require a rapid diagnosis (e.g. involvement of cardiac disease in dyspnoea (32)) or

chest pain) make this an exciting and rapidly expanding area of PCR development. If protocols can be developed to enable reverse transcription and PCR directly from patient samples (30)), this platform has the potential to make point-of care diagnostics based upon circulating microRNAs a reality.

Author contributions

WJA performed and analysed the experiments and assisted with manuscript preparation, EB and MD performed experiments with plasma and assisted with manuscript preparation, REH provided plasma samples and assisted with manuscript preparation, DAS conceived the study, analysed the data and wrote the manuscript.

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Competing Interests

The authors declare no competing interests.

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Tables

Table 1. Oligonucleotides.

	Name	Sequence (5'-3')	T_M (°C)
RT oligos	miR-21	UAG CUU AUC AGA CUG AUG UUG AAA AAA AAA AAA A	52.4
	OligodT-RACE	GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT TTT TTT TTT TTV N	61.9
	Reverse RACE	GCG AGC ACA GAA TTA ATA CGA C	53.9
18 S	18 S Forward	AAA CGG CTA CCA CAT CCA AG	55.3
	18 S Reverse	CCT CCA ATG GAT CCT CGT TA	53.8
miRNAs	miR-10b-5p	TAC CCT GTA GAA CCG AAT TTG TG	54.7
	miR-468-5p	TCC TGT ACT GAG CTG CCC CGA	62.8
	miR-126-5p	CAT TAT TAC TTT TGG TAC GCG	49.6
	miR-22-3p	AAG CTG CCA GTT GAA GAA CTG T	57.1
	miR-21-5p	TAG CTT ATC AGA CTG ATG TTG A	50.9
Y-RNAs	Y-RNA-3p	CCC CCA CTG CTA AAT TTG ACT	55.2
	Y-RNA-5P	GGC TGG TCC GAT GGT AGT	56.8

Sequence and melting temperatures for oligonucleotides used in this investigation. Column 1 denotes the general class of oligonucleotides used and Column 2 the name of each, also providing information about target strand (5p or 3p for miRNAs). Column 3 provides sequence information (V denotes any nucleotide not T or U and N denotes any nucleotide). Column 4 T_M values were calculated by Integrated DNA Technologies.

Table 2. PCR cycling conditions.

	xxpress			LightCycler480		
	Temp (°C)	**Hold (s)**	**Ramp rate (°C/s)**	**Temp (°C)**	**Hold (s)**	**Ramp rate (°C/s)**
Initial Denaturation	95	20	10 (4.8†)	95	20	4.8
Denaturation	95	1	10 (4.8†)	95	1	4.8
Annealing	60	10	10 (2.5†)	60	10	2.5
Extension*						
Cooling	50	10	10 (2.5†)	50	10	2.5

Cycling conditions for PCR. Ramp rates are shown for fast PCR and conventional PCR in parentheses. * Fluorescence was measured following each annealing and extension step. †Ramp rate used to assess consistency across the plate by amplification of 18S rDNA.

Figure legends

Figure 1. The xxpress system. A) 96 well xxplate (bottom) compared to conventional 96 well and 384 well PCR plates (upper). (Scale bar represents 25 cm to allow a direct size comparison). B) The xxpress system comprises a heat sealer (left), centrifuge (center) and cycler unit (right).

Figure 2. Variability in Ct values across plates on both systems. A single 18S rDNA PCR mastermix was distributed across 48 wells of both a Lightcycler 384 well plate and xxpress 96 well xxplate (5 µl reaction/well). PCR was performed with the same ramp rate on each thermal cycler (4.8 °C/s). Ct values are presented by row from well A1 (active wells are illustrated in Supplementary Figure.1). Melt curve analysis was performed to confirm amplification of a single product (data not shown).

Figure 3. Standard curves generated by amplification of 18S rDNA from genomic DNA and synthetic miR-21. A) 18S rDNA standard curve analysis. Comparable dynamic range 0.1 pg- 10 ng genomic DNA. Amplified using maximal ramp rate on each platform (n=3 technical replicates) (* $p < 0.05$, ** $p < 0.01$). B) Synthetic miR-21 standard curve analysis. Comparable dynamic range 10^9 - 10^6 copies of template RNA. Amplified using maximal ramp rate on each platform (n=3 technical replicates). For both datasets melt curve analysis confirmed the presence of a single product (data not shown).

Figure 4. Detection of microRNAs from human plasma samples by fast and standard PCR analysis. Cts for 4 microRNAs (miR-21, miR-22, miR-126, miR-486) and Y-RNA (Y-RNA-3p) on plasma samples from 3 individuals (n=3 technical replicates). The relative levels of expression detected by both systems were comparable. Gel and melt curve analysis was carried out to confirm amplification of one product (data not shown).

Supplementary Figure 1. Plate layout. Reported data is presented by row starting from well A1.